Running title: Shared genes for anorexia nervosa and obesity

Evidence for three genetic loci involved in both anorexia nervosa risk and variation of body mass index

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Abstract

Introduction The maintenance of normal body weight is disrupted in patients with anorexia nervosa (AN) for prolonged periods of time. Prior to the onset of AN, premorbid body mass index (BMI) spans the entire range from underweight to obese. After recovery, patients have reduced rates of overweight and obesity. As such, loci involved in body weight regulation may also be relevant for AN and vice versa.

Methods Our primary analysis comprised a cross-trait analysis of the 1000 single nucleotide polymorphisms (SNPs) with the lowest p-values in a genome-wide association meta-analysis (GWAMA) of AN (GCAN, Boraska et al., 2014) for evidence of association in the largest published GWAMA for BMI (GIANT, Locke et al., 2015). Subsequently we performed sex-stratified analyses for these 1000 SNPs. Functional *ex vivo* studies on four genes ensued. Lastly, a look-up of GWAMA-derived BMI related loci was performed in the AN GWAMA.

Results We detected significant associations (p-values < $5x10^{-05}$, Bonferroni corrected $p < 0.05$) for 9 SNP alleles at 3 independent loci. Interestingly, all AN susceptibility alleles were consistently associated with increased BMI. None of the genes (chr. 10: *CTBP2, chr. 19: CCNE1, chr. 2: CARF* and *NBEAL1;* the latter is a region with high linkage disequilibrium) nearest to these SNPs has previously been associated with AN or obesity. Sex-stratified analyses revealed that the strongest BMI signal originated predominantly from females (chr. 10 rs1561589; $p_{overall}$: $2.47 x10^{-06}$ / $p_{females}$: $3.45 x 10^{-07}$ / p_{males}: 0.043). Functional *ex vivo* studies in mice revealed reduced hypothalamic expression of *Ctbp2* and *Nbeal1* after fasting. Hypothalamic expression of *Ctbp2* was increased in diet induced obese (DIO) mice as compared to age-matched lean controls. We observed no evidence for associations for the look-up of BMI related loci in the AN GWAMA.

Discussion A cross-trait analysis of AN and BMI loci revealed variants at three chromosomal loci with potential joint impact. The chromosome 10 locus is particularly promising given that the association with obesity was primarily driven by females. In addition, the detected altered hypothalamic expression patterns of *Ctbp2* and *Nbeal1* as a result of fasting and DIO implicate these genes in weight regulation.

Keywords: obesity, loci, cross-disorder, shared, eating disorder, bulimia

Introduction

The joint analysis of GWAS data pertaining to different phenotypes/diseases with overlapping or co-morbid endophenotypes recently led to the discovery of novel genes that had escaped detection in single phenotype/disease analyses. For instance, by a cross-disorder analysis of five major psychiatric disorders common underlying biological mechanisms were revealed (Cross-Disorder Group of the Psychiatric Genomics Consortium et al., 2013; Serretti et al., 2013). A number of genetic variants were associated with more than one psychiatric disorder, illustrating the usefulness of the approach. Other cross-disorder analyses have shown overlapping genetic risk factors for phenotypes that had not been expected to share risk factors (e.g., ulcerative colitis and bone density or white blood cell count; Anderson et al., 2012). Heritability of anorexia nervosa (AN) is moderately high (for reviews see Gorwood et al., 2003; Helder and Collier, 2011; Thornton et al., 2011; Clarke et al., 2012; Hinney and Volckmar, 2013). However, the two published GWAMA were underpowered to detect signals (Wang et al. 2011; Boraska et al. 2014) to detect signals of small effect sizes, which are characteristic of SNPs identified for other psychiatric disorders (Cross-Disorder Group of the Psychiatric Genomics Consortium et al., 2013; Serretti et al., 2013). The largest GWAMA for AN was performed in 2,907 patients with AN and 14,860 controls by the Genetic Consortium for AN (GCAN) and the Wellcome Trust Case Control Consortium 3 (WTCCC3). Although a global meta-analysis comprised discovery and replication data sets on a total of 5,551 AN cases and 21,080 controls, genome-wide significance was not reached (Boraska et al., 2014). However, 76% of the variant effects were directionally consistent between discovery and replication groups. This observation was unlikely to be spurious ($P = 4 \times 10^{-6}$; Boraska et al., 2014).

A substantial genetic contribution to the variance of body mass index (BMI) is implicated by twin, family and adoption studies (Maes et al., 1997; Hinney et al., 2010). The largest currently published GWAMA pertaining to BMI variance revealed 97 genome-wide significant ($p \leq 5x10^{-08}$) gene loci (Locke et al., 2015); we use the term 'BMI SNPs' for those SNPs associated with an increased BMI. As most of the respective genes are expressed in the brain, a largely central regulation of human body weight appears likely (Willer et al., 2009; Locke et al., 2015). A region on chromosome 16p11.2 supports a possible genetic link between obesity and AN. Carriers of the respective deletion(s) are hyperphagic and obese, whereas the carriers of the duplication(s) are underweight and show restrictive / selective eating behavior (Walters et al., 2010; Jacquemont et al., 2011).

Sex-specific analyses have previously been conducted for BMI and related phenotypes. For instance, the weight increasing effect was more pronounced in female mice of the initial melanocortin-4 receptor gene (*Mc4r*) knock-out strain (Huszar et al., 1997). In humans with *MC4R* mutations leading to reduced function, the weight increasing effect was also stronger in females (Dempfle et al., 2004). Sex-stratified GWAMAs for waist-hip ratio variation and other anthropometric traits (height, weight, body mass index, waist circumference, and hip circumference) revealed a sexual dimorphism in the genetic effects for fat distribution and waist phenotypes (Heid et al. 2010; Randall et al. 2013, Shungin et al., 2015; Winkler et al., 2015). For many of these, genome-wide significance was detected for females only (Heid et al., 2010; Randall et al. 2013).

AN might be considered as an extreme weight condition (Hebebrand and Remschmidt 1995), potentially entailing that genetic factors involved in body weight regulation may overlap with those predisposing to AN as suggested by several groups (Hebebrand and Remschmidt 1995, Hinney et al., 2004, Pinheiro et al., 2006, Sulek et al., 2007, Day et al., 2009; Scherag et al., 2010, Boraska et

al., 2014; Gervasini and Gamero-Villarroel 2015). Recent LD-score regression analyses revealed a negative genetic correlation between AN and obesity (and a similar genetic correlation with BMI) suggesting that the same genetic factors influence normal variation in BMI as well as dysregulated BMI in AN (Bulik-Sullivan et al., 2015). However, in the latest GWAMA for AN 89 SNPs with genome-wide significance for BMI variation and obesity (Fall and Ingelsson, 2014; Guo et al., 2013) and 15 SNPs related to extreme obesity (Fall and Ingelsson, 2014) were not associated with AN (Boraska et al., 2014).

There is no evidence for an aberrant body weight regulation prior to manifestation of AN; thus, recalled premorbid weight of AN patients seemingly covers the whole BMI range (Coners et al., 1999; Föcker et al., 2015; Hebebrand, 2015). The BMI range of patients at medium term (five to ten years) follow-ups is shifted to the left (lower BMI); in recovered patients overweight occurs with a substantially lower probability than in the general population (Hebebrand et al., 1997; Keski-Rahkonen et al., 2014).

Here we performed three cross-trait analyses involving AN risk and BMI variation in two GWAMAs. First, we performed a cross-trait analysis of the 1000 SNPs with the lowest p-values from the largest GWAMA for AN (GCAN, Boraska et al., 2014) for evidence of association in the largest published GWAMA for BMI variation (GIANT; Locke et al., 2015). Second, we performed subsequent sensitivity analyses in sex-stratified data sets from the BMI GWAMA for the best cross-trait SNPs (Table 1) because of the profound female preponderance in AN (Steinhausen and Jensen 2015, Knoll et al., 2013); furthermore, sex-stratified analyses have revealed BMI loci that had not been detected in sex-combined analyses (Locke et al., 2015). Finally, we performed a

look-up of GWAMA derived BMI, (childhood) obesity and waist-hip ratio (WHR) loci within the AN GWAMA.

Post hoc we also performed (1) a look-up of the best cross-trait SNPs (Table 1) in: (1) obese children and adolescents from the EGG Consortium (Bradfield et al., 2012), and (2) the first GWAS for AN (Wang et al., 2011) comprising 1,033 AN cases and 3,733 paediatric controls from the Price Foundation Collaborative Group and the Children's Hospital of Pennsylvania. Finally, we performed functional studies of the four genes nearest to the best cross-trait findings.

Materials and Methods

Look-up of 'AN SNPs' (GCAN) in GIANT GWAMA for BMI including sex-specific analyses: Our primary analysis is based on the *in silico* look-up of the 1000 best hits according to p-value (SNPs in high linkage disequilibrium (LD) were not excluded) derived from the case-control AN GWAMA (Boraska et al., 2014) in the large scale GWAMA of up to 322,135 individuals from the population-based GIANT meta-analysis for BMI (Locke et al., 2015). In light of the aforementioned results for obesity risk alleles in the AN GWAMA (Boraska et al., 2014), we did not pursue the directional hypothesis that AN susceptibility / risk alleles are protective of obesity (i.e. are expected to be BMI lowering); as a consequence, we report two-sided tests. Secondarily, we performed sex-stratified analyses for the best cross-trait SNPs in the BMI GWAMA (Locke et al., 2015).

We estimated the percentage of AN GWAMA SNPs that met the same p-value threshold in the BMI GWAMA (Supplementary Figure S1). We estimated that the genetic overlap of BMI and AN can seemingly be demonstrated if the number of SNPs analyzed is larger than 500, so that the 1000 SNPs we had chosen is justified. We decided against a computational derivation of an

"optimal" cut-off as this could inflate the type I error rate. We did not aim for a comprehensive assessment of the joint common SNP variation architecture of both traits.

Post hoc we performed analyses in the sub data sets of GIANT (*a:* full GWAS chip data on N~233,000; *b:* Metabochip on N~88,000; supplementary Table S7) to analyze if the observed effects are confirmed for each sub data set.

Look-up of 'BMI SNPs' in GWAMA for AN susceptibility (GCAN): We performed an *in silico* look-up of the 56 novel genome-wide significant `BMI SNPs´ detected by Locke et al. (2015) in the case-control AN GWAMA (GCAN; Boraska et al., 2014). Subsequently we also analyzed previously described SNPs for BMI, obesity, childhood obesity and WHR (2015, see supplementary Tables).

A total of 2,916 quality controlled genotypes of controls were included in both GCAN and GIANT (n=1,437 NBS-WTCCC National Blood Service donors and n=1,479 British 1958 birth cohort-WTCCC). This is a minor percentage only (0.86% for GIANT; 19.6% for GCAN controls) of the analyzed individuals in total and we decided against a re-analysis excluding these overlapping samples.

Subsequent look-ups in independent GWAS data sets: We performed a look-up of the best cross-trait SNPs in GWAMA data of the EGG Consortium (Bradfield et al., 2012) consisting of 5,530 obese children and adolescents (BMI ≥95th percentile) and 8,318 controls (BMI <50th percentile). Data on the childhood obesity trait has been contributed by the EGG Consortium and was downloaded from www.egg-consortium.org (Bradfield et al., 2012).

An additional look-up of the best cross-trait SNPs from GCAN and GIANT was performed in the first GWAS for AN (Wang et al., 2011) consisting of 1,033 AN cases and 3,733 paediatric control subjects of European ancestry; five SNPs were available.

Written informed consent to take part in genetic association studies was given by all participants and in case of minors by their parents. Studies were approved by the respective institutional review boards or ethics committees and conducted in accordance with *The Declaration of Helsinki* (Bradfield et al., 2012; Wang et al., 2011; Boraska et al., 2014, Locke et al., 2015).

Statistical analyses: We performed two main analyses and one stratified analysis nested within the first main analysis focussing on European-descent individuals of two GWAMAS. The GWAMA for AN (Boraska et al., 2014) was performed as fixed-effect meta-analysis based on single-SNP case-control association analyses under an additive genetic model with control for population stratification at the discovery data set level. Similarly, the GWAMA for BMI variation (Locke et al., 2015) also worked with a fixed-effect meta-analysis based on discovery data set results obtained under a linear regression model adjusted for age, age^2, sex and study-specific covariates including control for population stratification effects. For the first main analysis, we looked-up the 1000 SNPs of the GWAMA for AN (Boraska et al., 2014) with the lowest p-values (discovery p-values from 5.56×10^{-22} to 4.79×10^{-04}, of note: the SNP with the lowest p-value in the initial discovery GWAS for AN was not confirmed by genotyping in the replication sample) in the GWAMA for BMI (Locke et al., 2015). We applied a conservative Bonferroni-correction to the uncorrected p-values of the GWAMA for BMI to address multiple testing (see Table 1 and Supplementary Tables), and accordingly regarded all associations as significant which met a nominal p-value $\leq 5 \times 10^{-5}$ (Table

1). For the SNPs with significant associations in the GWAMA for BMI, we also report the results of sex-stratified sensitivity analyses (Table 1). For the second main analysis, we performed a look-up of the 97 BMI loci in the GWAMA for AN (see supplementary Tables S1-S3). The direction of effect was evaluated only for SNPs with a nominal p-value ≤ 0.05.

Post hoc we also analyzed genome-wide significant loci for BMI, obesity, childhood obesity not originally described in Locke et al., 2015 (reviewed in Yazdi et al. 2015; Supplementary Table S4) and 68 genome-wide significant loci for waist-hip ratio (WHR) derived from a European GWAMA primary analysis (GIANT, Shungin et al., 2015; Supplementary Table S5) in the GWAMA for AN (GCAN, Boraska et al., 2014).

***Animals and diet*:** Unless stated otherwise, male C57BL/6J mice were fed *ad libitum* with either a standard chow diet (Harlan Teklad LM-485; 5.6% kcal fat) or a high-fat diet (D12331; Research Diets, New Brunswick, NJ; 58% kcal fat). The mice had free access to water and were maintained under constant ambient conditions (22 ± 1°C, constant humidity, 12h/12h light/dark cycle). All animal studies were performed in Cincinnati, OH, USA and were approved by the Animal Ethics Committee of Cincinnati, OH, USA.

Gene expression analyses: To assess effects on fasting and re-feeding, hypothalamic gene expression was profiled in male 27/28 week old C57BL/6J mice fed either *ad libitum* with a regular chow diet, or which had been fasted for 12h, 24h, or 36h, or which had been fasted for 36h and then re-fed for 6h using either a fat-free diet or a high-fat diet (N=6-8 mice per group). The use of existing *ex vivo* material is in agreement with the US and German guidelines of the Animal Welfare

Committee to restrict animal experiments to an absolutely necessary minimum. Target genes were amplified using the ViiA 7 real-time PCR system (Life Technologies; Darmstadt, Germany); results were normalized to the housekeeping gene hypoxanthine guanine phosphoribosyltransferase 1 (HPRT). The used primer sequences were CTBP2-F: 3'- TACCACACCATCACCCTCAC -5'; CTBP2-R: 3'- TGTGGCAGACTGTCGAATCT-5'; CCNEI-F:3'-AGCCTCGGAAAATCAGACCA-5'; CCNEI-R: 3'-CTTCGCACACctccattagc-5', CARF-F: 3'-GTGGACGACAGATAGTGGGA-5'; CARF-R: 3'-GGAGAGGAGAGTCTTGGCTG-5'; NBEAL1-F: 3'-AGGAGAAGGAAATGGCTGATCA-5', and NBEAL1-R: 3'- TCCACTGTGAGAGAAGCTGG-5'. Data represent means ± SEM. *P<0.05, **P<0.01, based on a one-way ANOVA with Dunnett's Multiple Comparison post-hoc test.

To additionally assess the effects of a high fat diet on hypothalamic expression of *NBEAL1* and *CTBP2* was assessed in age matched male C57BL/6J mice fed either a regular chow diet (body weight 32.69g ± 0.45g) or a high-fat diet (body weight 54.72g ± 1.25g; N=7-8 mice per group.). Data represent means ± SEM.

***In silico* analyses**: Expression patterns and known variants in the coding regions (missense, nonsense and frameshift) were analyzed *in silico* (http://www.genecards.org/; http://exac.broadinstitute.org/about).

Results

Association of AN risk SNPs with increased BMI: We detected association (p-values < $5x10^{-05}$, Bonferroni corrected $p < 0.05$) at three independent chromosomal loci in the BMI GWAMA (chromosome 2: four SNPs in linkage disequilibrium [LD], $r^2 \geq 0.819$, D' = 1; chromosome 10: three SNPs, $r^2 \geq 0.363$, D' ≥ 0.728; and chromosome 19: two SNPs, $r^2 = 1$, D' = 1); the lowest p-value (rs1561589, 2.47×10^{-06}, $p_{corrected} = 0.0025$) was observed at the chromosome 10 locus (Table 1). Within the GIANT (Locke et al., 2015) data we *post hoc* also analyzed the data sets separately for (a) full GWAS chip data (HapMap imputed) on N~233,000 and (b) Metabochip on N~88,000 (Supplementary Table S7). Both independent data sets confirmed the association of the 9 SNPs.

The nearest genes to these nine SNPs ordered from lowest to highest p-values are: (1) chromosome 10: *CTBP2* (C-terminal binding protein 2 gene); (2) chromosome 19: *CCNE1* (cyclin E1 gene); (3) chromosome 2: *CARF* (calcium responsive transcription factor gene) and (4) *NBEAL1* (neurobeachin-like 1 gene). The third chromosomal locus included two genes, because the four SNPs are located in a region with high linkage disequilibrium (lowest LD for the four SNPs: $r^2 \geq 0.819$, D' = 1). Interestingly, for all SNPs, the AN risk alleles were consistently associated with increased BMI (Table 1).

Sex-specific analyses for the best cross-trait SNPs (Table 1) revealed that the chromosome 10 association signal was majorly triggered by females. Again, *post hoc* sex-specific analyses in the sub data sets of GIANT (*a:* full GWAS chip data on N~233,000; *b:* Metabochip on N~88,000; supplementary Table S7) confirmed the larger effect in females for the best locus.

Further look-ups: Because AN typically manifests during adolescence, we analyzed the identified SNPs in the EEG Consortium data set (Bradfield et al., 2012), which includes only children and adolescents. The look-up of the nine cross-trait SNPs (Table 1) did not reveal significant findings

at the five SNPs available (p-values 0.0916 at rs11245456 to 0.6075 at rs1561589). However, the direction of effect was the same between AN risk and early onset extreme obesity in all five SNPs.

The look-up of the nine cross-trait SNPs in the first GWAS for AN (Wang et al., 2011) comprising 1,033 AN cases and 3,733 paediatric controls (five SNPs were available, each locus was represented) showed nominally significant results for two SNPs at chromosome 2 (rs17406900; nominal p = 0.03; rs7573079; nominal p = 0.04), our second best locus. However, for these SNPs the direction of effect was opposite to the effect in GCAN.

Association of 'BMI SNPs' with AN: The look-up of the 'BMI SNPs' in the AN GWAMA did not reveal (Bonferroni-corrected for 97 SNPs) significant results (Supplementary Tables S1-S3). Similarly, *post hoc* lookups of additional genome-wide significant loci for BMI, obesity, childhood obesity (reviewed in Yazdi et al. 2015; Supplementary Table S4) and WHR (Shungin et al., 2015; Supplementary Table S5) in the GWAMA for AN (GCAN, Boraska et al., 2014) did not reveal statistically significant findings after correction for multiple testing.

In silico analyses: All four genes located at the three identified loci are widely expressed in brain tissues, including the hypothalamus (http://www.genecards.org/). A spectrum of different, potentially functionally relevant variants (missense, nonsense and frameshift) was detected for all four genes (Supplementary Table S6).

Mouse model: Gene expression profiling of *Ctbp2, Ccne1*, *Carf*, *Nbeal1* and in male C57BL/6J mice revealed that hypothalamic expression of both *Ctbp2* and *Nbeal1* was decreased by fasting (one-way ANOVA $p<0.05$ for both targets; Figure 1). Notably, hypothalamic expression of *Ctbp2* and *Nbeal1* remained decreased after 36h fasting followed by 6h re-feeding with either a fat-free diet or a high-fat diet relative to control mice fed *ad libitum* (Figure 1). In line with the down-regulation

of hypothalamic expression of *Ctbp2* and *Nbeal1* in response to nutrient availability, expression of *Ctb2* was increased in diet-induced obese compared to age-matched lean control mice ($p<0.01$; Figure 2); for *Nbeal1* we noted a trend for increased expression in obese compared to lean mice ($p=0.070$; Figure 2).

Discussion

Among the 1000 SNPs with the lowest p-values in the GCAN GWAMA for AN (Boraska et al., 2014) we identified nine SNPs in three chromosomal regions with significant p-values in the currently largest GWAMA for BMI variation (Locke et al., 2015) using a conservative Bonferroni correction.

The relevance of these three loci is uncertain, because none of the nine SNPs have previously been identified for either AN or BMI /obesity or other psychiatric disorders. Two *NBEAL1* SNPs (intronic and 3'UTR, rs16839626, rs6733725; no detectable LD to the SNPs identified here; http://www.broadinstitute.org/mpg/snap/ldsearchpw.php) had been detected in a GWAS for obesity related traits in 815 Hispanic children from 263 families. Nominal associations (not genome-wide significant) for energy storage and fat mass deposition ($p=2x10^{-07}$), fat mass change ($4x10^{-07}$) and weight change (3×10^{-06}) were shown (Comuzzie et al., 2012). Central (including hypothalamic) expression of all four genes was detected. We did not detect association of the previously published GWAMA SNPs for BMI, (childhood) obesity, or WHR with AN (Supplementary Tables).

The following results do not readily substantiate the relevance of our association findings: a) The analysis of the five out of nine available cross-trait SNPs in 5,530 obese children and adolescents

(BMI ≥95th percentile) versus 8,318 controls (BMI <50th percentile) from the EGG Consortium (Bradfield et al., 2012) did not reveal significant findings. However, for all available SNPs the direction of effect was identical to that observed in the GIANT GWAMA. Because the EGG Consortium GWAMA is substantially smaller than the recent GIANT approach (Locke et al., 2015), true signals may not have been detectable. b) The look-up of the same cross trait SNPs in the first GWAS for AN (Wang et al., 2011) did not support our findings. This might partly be explained by the lower sample size in the analysis of the Price Foundation Collaborative Group and Children's Hospital of Pennsylvania samples (1,033 AN cases and 3,733 pediatric controls in Wang et al., 2011) compared to the latest GWAS (2,907 cases with AN and 14,860 controls in Boraska et al., 2014). In conclusion, we cannot exclude that our detected associations for the nine SNPs represent false positive associations.

The following lines of evidence do however support that we have indeed detected SNPs associated with both anorexia nervosa and obesity:

The identification of the three loci with nominal p-values in the range of 10^{-5} to 10^{-6} for association with BMI is quite unexpected. Accordingly, at least one and maximally all three loci are involved in body weight regulation; the same potentially holds true for AN. If this assumption is correct, future larger GWAMAs for both AN and BMI/obesity will pick up the respective loci. It is also of interest that all risk alleles were directionally consistent for AN risk and higher BMI. This is especially unexpected as patients with AN do not have an elevated premorbid BMI (Coners et al., 1999); in addition, BMI-values of followed up patients only infrequently exceed the cutoff for overweight (BMI ≥ 25 kg/m²; Hebebrand et al., 1997). It is unlikely that the small sample overlap between the AN (controls) and GIANT GWAMAs explains our results.

Sex-specific analyses: We also performed look-ups in sex-stratified analyses for the best cross-trait SNPs in the BMI GWAMA, because (i) AN predominantly occurs in females (Steinhausen and Jensen 2015; Knoll et al., 2013) and (ii) sex-specific analyses rendered BMI loci that had not been picked up by sex-combined analyses (Locke et al., 2015). We found that the three AN risk SNPs at the chromosome 10 locus with the lowest p-values in the BMI GWAMA (sex-combined) mainly originated from the female participants (Table 1). This finding provides additional indirect evidence that particularly this locus is involved in both AN and body weight regulation in females mainly.

Animal model The hypothalamic expression data obtained in male mice clearly substantiate that the detected associations at two loci may indeed represent true positive findings. The cDNA of the fasting/re-feeding experiment described in this manuscript is commonly used in the Müller/Tschöp lab to assess regulation of target genes. Whereas there is unfortunately no documentation on the total number of so far analyzed targets, it can be confirmed that only few of the so far analyzed genes have been found to be differentially regulated under the here reported conditions (e.g. Müller et al., 2013). Expression of *CtBP2*, whose locus represented our strongest association signal (Table 1), proved to be inversely regulated by fasting and diet induced obesity. Thus, hypothalamic gene expression was reduced for this gene and additionally in fasted (12, 24 or 36 h) mice; this down-regulation persisted 6 hours after renewed access to *ad-libitum* feeding (re-feeding for 6 h with either a high-fat diet or a fat-free diet). Genes, whose expression is down-regulated in fasting, are usually anorexigenic (e.g. leptin: Müller et al., 2009, Hebebrand et al., 2007), while expression of orexigenic genes (e.g. ghrelin: Müller et al., 2015) is increased in

fasting. Hence it is likely that both Ctbp2 and Nbeal1 have an anorexigenic effect. In accordance with this assumption, both genes were up-regulated in diet induced obesity (DIO; Figure 2).

BDNF signalling: It is of interest to point out that the two genes *CTBP2* and *CARF* are involved in BDNF signaling pathways (Tong et al., 2013; Shieh et al., 1998; Shieh and Ghosh 1999; Finkbeiner et al. 2000; Xia et al., 2002; Tao et al., 2002; Williams et al., 2003; Merighi et al., 2008; Singer et al., 2008; McDowell et al., 2010; Pfenning et al., 2010; Alboni et al., 2010; West 2011; Lyons et al., 2012; Calabrese et al., 2013a,b; Ji et al., 2014); the leptinergic-melanocortinergic-BDNF pathway includes genes with known genetic variation underlying both monogenic and polygenic obesity (Hinney et al., 2014). Multiple SNPs near *BDNF* are genome-wide significantly associated with obesity (e.g. Speliotes et al., 2010; Locke et al., 2015). Evidence for an involvement of BDNF in AN stems from studies on (a) animal models, (b) genetics, and (c) serum or brain levels of BDNF. However, some of the data are equivocal. In more detail: (a) in animal models the central infusion of BDNF induces weight loss (Pellymounter et al., 1995; Bariohay et al., 2005). The suppressive effects of BDNF on feeding behavior and body weight is mediated by corticotropin-releasing factor (CRF) and hypothalamic neuronal histamine in mice (Gotoh et al., 2013). BDNF signaling is altered by reduced BDNF expression in the hippocampus, in activity-based anorexia in mice (Gelegen et al., 2008) and in immobilization stress induced anorexia in rats (Charrier et al., 2006). Deletion of the *Bdnf* gene in the PVH resulted in hyperphagia, reduced locomotor activity, impaired thermogenesis, and severe obesity. Additionally, in response to cold exposure BDNF expression in the PVH was increased (An et al., 2015). (b) Association of variation in BDNF with AN was shown by some but not all studies (Ribasés et al., 2003, 2004, 2005; de Krom et al., 2005; Friedel et al., 2005; Dardennes et al., 2007; Rybakowski et al., 2007; Dmitrzak-Weglarz et al., 2007;

Slof-Op 't Landt et al., 2011; Ando et al., 2012; Brandys et al., 2013; Pjetri et al., 2013; Gamero-Villarroel et al., 2014). For the widely studied *BDNF* Val66Met variant, a recent meta-analysis showed no association of the infrequent 66Met allele with AN (Brandys et al., 2013). (c) Decreased serum and brain levels of BDNF had unequivocally been reported in patients with AN (Pelleymounter et al., 1999; Nakazato et al., 2003, 2006, 2009, 2012; Monteleone et al., 2004, 2005, 2013; Mercader et al., 2007a, b, 2010; Ehrlich et al., 2009; Saito et al., 2009; Brandys et al., 2013; Dmitrzak-Weglarz et al., 2013; Zwipp et al., 2014; Eddy et al, 2015). This was recently confirmed in a meta-analysis (Brandys et al., 2011). While only one study has hinted at an interaction between CTBP2 and BDNF (Tong et al., 2013), the interaction of CARF and BDNF has been substantiated in numerous studies (Shieh et al., 1998; Shieh and Ghosh 1999; Finkbeiner et al. 2000; Xia et al., 2002; Tao et al., 2002; Williams et al., 2003; Merighi et al., 2008; Singer et al., 2008; McDowell et al., 2010; Pfenning et al., 2010; Alboni et al., 2010; West 2011; Lyons et al., 2012; Calabrese et al., 2013a,b; Ji et al., 2014). Thus, again as BDNF might be involved in both AN and obesity (reviewed in Day et al., 2009; Rask-Andersen et al., 2010; Rosas-Vargas et al., 2011), this gene is biologically highly plausible. One study hinted at an interaction between CTBP2 and BDNF (Tong et al., 2013

In the following we provide additional information on the genes located nearest to the three loci identified via the nine SNPs starting with the chromosome harboring the SNPs with the lowest p-values:

Chromosome 10: The three intronic SNPs in the *CTBP2* gene (C-terminal binding protein 2) show the lowest p-values in our BMI GWAMA (Locke et al., 2015) look-up (Table 1); as stated above the effect is almost only due to females. The two alternative *CTBP2* transcripts lead to two distinct

proteins, one of which is a transcriptional repressor, while the other is a major component of synaptic ribbons, a specialized form of synapses. A NAD+ binding domain is common to both isoforms. There is evidence that the gene/protein is involved in brown adipose tissue function and regulation (Banke et al., 2014; Chornokur et al., 2013; Guirguis et al., 2013; Vernochet et al., 2009, 2010; Farmer 2008; Kajimura et al., 2008). *Ctbp2* knock-out mice displayed abnormal phenotypes in the cardiovascular and central nervous systems, in addition to having effects on embryogenesis, growth/size/body, and mortality / aging (http://www.informatics.jax.org/allele/MGI:2183646; Hildebrand and Soriano 2002). Recently, a miRNA that was upregulated during the development of obesity in mice (miR-342-3p) was described to promote a suppressing effect on CtBP2 (Wang et al. 2015), again underscoring the relevance of the gene for weight regulation.

Chromosome 19: The cyclin E1 gene (*CCNE1*) identified via the two SNPs 5´ to this gene encodes a protein that belongs to the highly conserved cyclin family. Cyclins act as (i) regulators of specific kinases and (ii) contribute to the coordination of mitotic events. In many tumors overexpression of this gene has been observed (Li et al., 2015). It was recently shown that proliferation of 3T3-L1 preadipocytes promoted by recombinant myostatin increased expression of proliferation related genes (e.g. cyclin E1) by 20.5 %; Zhu et al., 2015).

Chromosome 2: The third chromosomal locus includes two genes, because the four SNPs are located in a region with high linkage disequilibrium (lowest LD for the four SNPs: $r^2 \geq 0.819$, D' = 1). Three of the SNPs are located in an intron, one is 5' to *NBEAL1* (see Table 1): (1) The calcium-response factor gene (*CARF* or as an alias name amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 8 gene: *ALS2CR8*) acts as a transcriptional activator that mediates the calcium- and neuron-selective induction of BDNF expression (Tao et al., 2002). Lack of *Carf*

(*Als2cr8*) in knock out mice results in deficits associated with learning and memory (McDowell et al., 2010). Functionally relevant recessive mutations in the gene have been described in patients with amyotrophic lateral sclerosis 2 (ALS2; Hadano et al., 2001).

In sum, in a cross-trait analysis for genetic loci involved in AN risk and increased BMI three chromosomal loci with potential relevance for both traits were detected. Apart from the identification of these loci, their role in both AN and body weight regulation was particularly substantiated by *ex vivo* data of mouse models for fasting and DIO suggesting an anorexic role of CTBP2 and NBEAL1, by the sex specific results for CTBP2 and the finding that CTBP2 and CARF are involved in *BDNF* regulation. Further in depth molecular genetic and biological analyses are essential to understand the relevance of these loci and the genes they contain in the etiology of AN and in body weight regulation/obesity. The association of AN alleles with increased BMI might imply that a specific genetic variant (allele) can either increase or decrease BMI depending on presence or absence of additional factors with an influence of the body weight (e.g. occurrence of an eating disorder). If true, this general concept has implications for all gene mapping approaches in genetic epidemiology calling for more hypothesis-driven stratified analyses.

A spectrum of different variants (missense, nonsense and frameshift) has been described for the four genes (Supplementary Table 3), so that a mutation screen in these genes in study groups of patients with AN or extreme obesity is warranted.

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Conflict of Interest

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Figure 1

Hypothalamic expression of *Ctbp2* (a), *Ccne1* (b), *Carf* (c), *Nbeal1* (d), or in response to fasting for 12, 24 or 36 h, and after re-feeding for 6 h with either a high-fat diet (HFD) or a fat-free diet (FFD, N 1⁄4 6–8 mice per group) (c). *P<0.05, **P<0.01, based on a one-way ANOVA with Dunnett's Multiple Comparison post-hoc test

Figure 2

Hypothalamic expression of Ctbp2 and Nbeal1 in diet induced obesity (DIO) as compared to age-matched lean control mice.

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